NUTRITIONAL AND ENVIRONMENTAL FACTORS AFFECTING GLYCEROL PRODUCTION BY *ASPERGILLUS WENTII* IMI 023010

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ABSTRACT - *Aspergillus wentii* IMI 023010 successfully produced high level of glycerol on a chemically defined liquid medium. Biosynthesis of glycerol was markedly affected by composition of culture medium, pH and temperature as well as incubation period. Medium of the following composition was favourable for the production of glycerol: NaCl, 150g; mannitol, 10g, $NaNO_3$, 2g; KH_2PO_4, 1g; $ZnSO_4$, 0.22 mg; $CaCl_2$, 0.14 mg and casamino acids, 6 g (per liter of distilled H_2O). Production of glycerol was maximum at pH 6-7 and after 15 days of incubation at 20°C.

RÉSUMÉ - *Aspergillus wentii* IMI 023010 produit, sur milieu liquide chimiquement défini, du glycérol en grande quantité. La biosynthèse du glycérol est affectée par la composition du milieu, le pH, la température ainsi que par la durée d'incubation. La composition du milieu favorable à la production de glycérol est: NaCl, 150 g, mannitol, 10g, $NaNO_3$, 2g; KH_2PO_4, 1g; $ZnSO_4$, 0.22 mg; $CaCl_2$, 0.14 mg; caséine, 6 g (pour 1l d'eau distillée). La production du glycérol est maximale à pH 6.7, après 15 jours de culture à 20°C.

KEY-WORDS - Glycerol production, filamentous fungi, *Aspergillus*.

INTRODUCTION

Glycerol as a by-product in the manufacture of soap and fatty acids does not promise its supply. Today, glycerol is typically produced chemically from petroleum derivatives which is less expensive than processing by sugar fermentation. With the chemical synthesis of glycerol (Neumuller, 1981), environmental problems can arise because of possible evolution of chlorinated by-products in the course of production process.

Recently there has been a great revival in the interest in glycerol fermentation in the world due to rapidly diminishing petroleum reserves (Vijaikishore & Karanth, 1986). Many attempts have been made to improve biotechnological glycerol production for commercial application with the main emphasis on high glycerol concentrations in the product both at rapid fermentation rates and low process and energy costs (REHM, 1988).

Glycerol is formed by several yeasts at higher osmotic potential in the medium to protect enzymes and maintain normal cell turgor (Brown et al., 1986). However, little is known concerning glycerol production by filamentous fungi. Thus, the objective of this investigation was designed to study glycerol production by *Aspergillus wentii* IMI 023010 (known as a glycerol producer) under different environmental and cultural conditions.

MATERIALS AND METHODS

Organism

Aspergillus wentii (IMI 023010), known as a glycerol producer was obtained from the International Mycological Institute, Kew, Surrey, England.

Cultivation

The fungus was inoculated into autoclaved (121°C, 20 min, 15 lb/in^2) 250 ml Erlenmeyer flask containing 50 ml of the desired medium. After inoculation with 2 ml inoculum suspension of 2 weeks old culture of the experimental organism, the flasks were incubated at 28°C or other mentioned temperatures for 15 days without shaking.

Cultural condition

Czapek's medium with 10 g glucose and 2 g $NaNO_3$ (per liter) as carbon and nitrogen source, respectively and fortified by 15% NaCl was used as starting culture medium. The effect of carbon and nitrogen sources on glycerol production were determined by replacing glucose or $NaNO_3$ with equimolar concentrations of test compounds. Certain metal were added as salts to the defined cultural medium at a concentration of 25 µmol/L to determine their influence on glycerol production by the experimental organism. Aliquots of the culture medium were adjusted before sterilization to the desired pH values using N/10 of both HCl and NaOH. All experiments were done in triplicate and yields were reported as averages.

Glycerol analysis

Glycerol was analyzed enzymically using a commercially enzyme combination (Boehringer, Mannheim glycerol test combination catalogue no. 148270) based on the method of Eggstein & Kuhlmann (1974). Readings were made with a Spectronic 2000 at 340 nm. Results were recorded as µ mol of glycerol per ml of culture medium.

RESULTS AND DISCUSSION

Attempts have been made to evaluate the effect of different carbon sources and the suitable concentration of the most favourable carbon source for both supporting fungal growth and stimulating glycerol production by *Aspergillus wentii* IMI 023010. Nine different carbon sources were tested. Lactose, maltose and soluble starch permitted weak growth and low levels of glycerol (Fig. 1). Inlow et al. (1988) reported that glycerol production by *Saccharomyces cerevisiae* was significantly decreased when

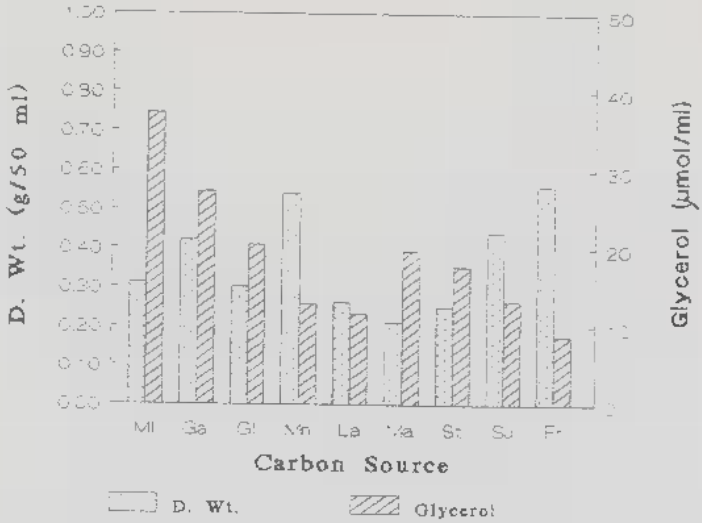

Fig. 1: Effect of different carbon sources on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 1: Effets de différentes sources carbonées sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

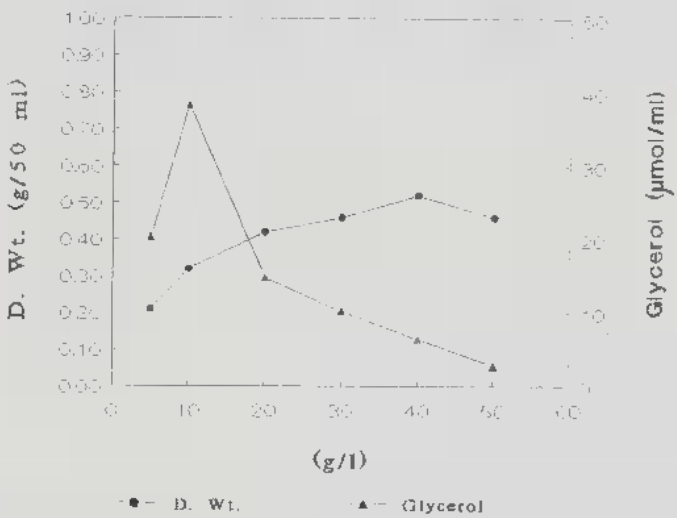

Fig. 2: Effect of different mannitol concentrations on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 2: Effets de la concentration en mannitol sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

soluble starch was used instead of glucose. Fructose, mannose and sucrose supported fungal growth, but low levels of glycerol were obtained. Galactose followed by glucose were stimulators for both mycelial growth and glycerol production while mannitol was superior for glycerol production by the isolate tested (Fig. 1). Maximum glycerol synthesis was obtained at 10 g of mannitol per liter (Fig. 2). Earlier results obtained by Onishi & Susuki (1968) showed that glucose followed by galactose gave the highest yield of glycerol by a unidentified yeast strain isolated from soy-sauce mash and a glucose concentration of 30% increased glycerol yield. Recently Hendriksen et al. (1988) reported that in high osmotic cultures with both sucrose and lactose higher yield of glycerol was obtained by *Penicillium scabrosum*.

The experimental organism was able to utilize a wide range of nitrogen sources. All inorganic nitrogen sources tested (except $NaNO_2$) were utilizable and suitable for mycelial growth. Nitrate was more suitable for production of glycerol. Sodium nitrate proved to be more favourable than any other nitrogen sources tested for production of glycerol by the experimental organism (Fig. 3). Increasing the nitrogen level of culture medium induced parallel increase of mycelial growth. Glycerol synthesis by the experimental organism reached its maximum at a concentration of 2 g of sodium nitrate per liter (Fig. 4). These results are not in harmony with that previously obtained by Onishi (1959). He reported that when an inorganic ammonium salt was used as nitrogen source, the rapid decrease in the pH of the medium during the fermentation might reduce polyols yield. On the other hand, Onishi & Susuki (1968) found that both ammonium nitrogen (as ammonium sulfate and ammonium lactate) and amino nitrogen (as sodium L-glutamate) were found to be available for mannitol and glycerol production by an isolate of yeast. However, Ross & Morris (1962) working with marine yeasts, which would probably have accumulated glycerol, found that the type of nitrogen source used in the medium did not affect their ability to tolerate high NaCl concentrations. Holligan & Jennings (1972) recorded high activity of the pentose phosphate pathway and hence the amount of glycerol formed is higher on nitrate media than on asparagine media. Recently, Luard (1985) studied the difference between growth of four fungi on organic and inorganic nitrogen and found that greater yield of growth and glycerol were obtained on organic nitrogen.

Variation of KH_2PO_4 content from 0 to 2 g/l did not affect glycerol production by the experimental organism, while the higher levels (5 and 10 g/l) reduced glycerol accumulation (Fig. 5). These results are in harmony with results previously recorded by Spencer & Shu (1957) and Peterson et al. (1958). They reported that the excess of inorganic phosphate has detrimental effect upon the yield of polyalcohol in some yeasts. However, Onishi & Susuki (1968) recorded that inorganic phosphate level did not affect either glycerol or mannitol production by yeasts.

The pH value of the nutritive medium exerted a marked influence on glycerol production. Using initially adjusted medium, the results (Fig. 6) revealed that mycelial growth and glycerol biosynthesis increased gradually with the increase of pH values reaching maxima at pH ranging from 6.0 to 7.0 for the organism tested. The growth of fungi under conditions where water availability is a limiting factor is controlled primarily by the water activity (a_w) (Scott, 1957), although other factors such as pH of the medium and the solutes present are also known to be influential (Pitt, 1975).

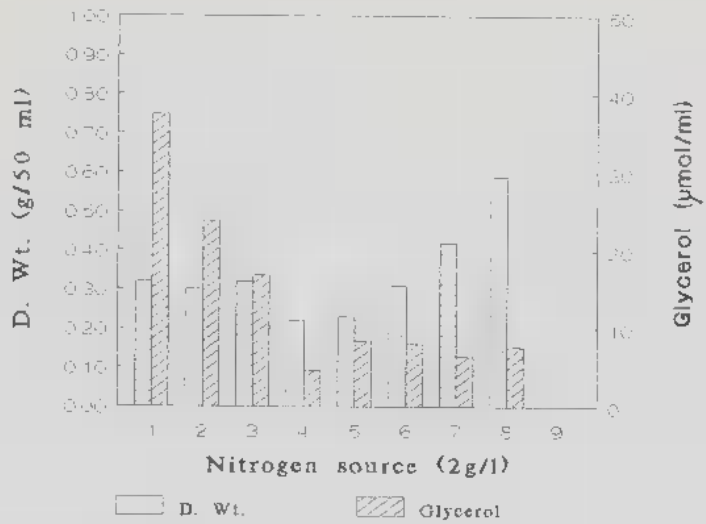

Fig. 3: Effect of different nitrogen sources on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 3: Effets de différentes sources azotées sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

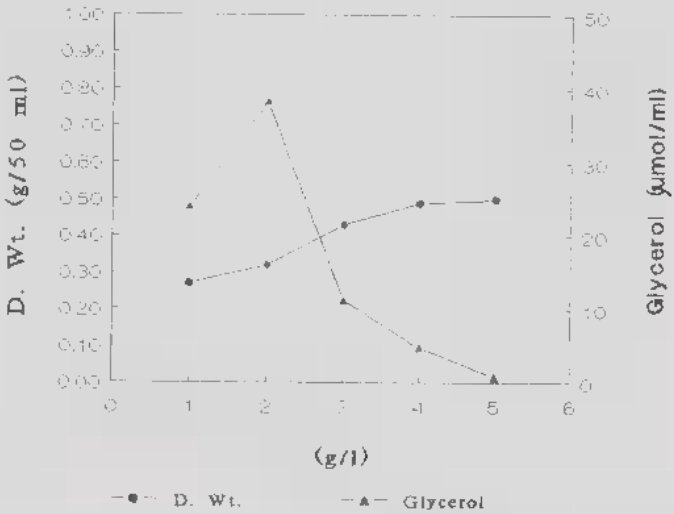

Fig. 4: Effect of different $NaNO_3$ concentrations on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 4: Effets de la concentration en $NaNO_3$ sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

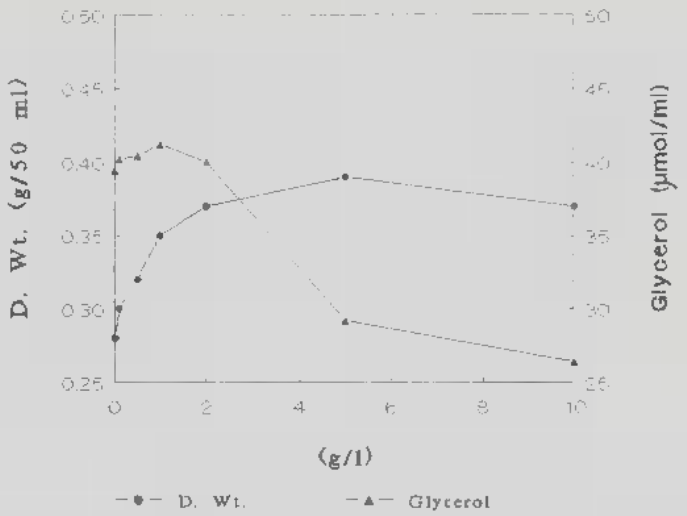

Fig. 5: Effect of different concentrations of KH_2PO_4 on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 5: Effets de la concentration en KH_2PO_4 sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

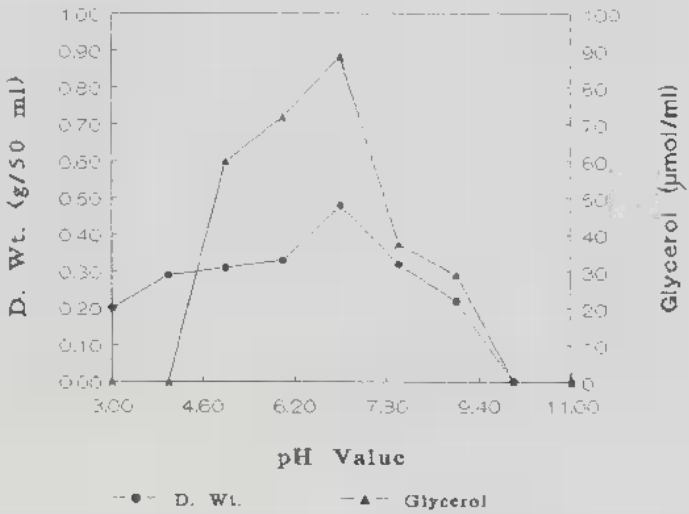

Fig. 6: Effect of pH on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 6: Effet du pH sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

Available data are fragmentary and contradictory, Parekh & Pandey (1985) reported that pH control did not affect glycerol production by *Hansenula anomala*. Hendriksen et al. (1988) studied polyols formation by *Penicillium scabrosum* and reported that the yield of mannitol was higher in cultures with neutral pH than in cultures with a slightly acidic pH; for glycerol the reverse was the case. Also, Abdel-Hafez (1991) indicated that maximal glycerol production by *Eurotium amstelodami* was attained at pH 5, at which maximum of mycelial growth was also recorded.

Incubation temperature proved to be an important factor in glycerol synthesis. 30°C and 20°C were the optima for mycelial growth and glycerol accumulation by *Aspergillus wentii* (Fig. 7). According to Spencer et al. (1957), the effects of temperature and initial sugar concentration are interrelated. The found that the cultures of osmophilic yeasts produce low levels of glycerol when yeast is grown at 30°C in media of low sugar concentration. When higher concentration of carbon and nitrogen sources were used, the maximum temperature for growth and glycerol production was 37°C. It has been previously noted that tolerance of different fungi to salt was increased with the increase in the temperature of incubation (Gold, 1959). Recently, Abdel-Hafez (1991) reported that the maxima of mycelial growth and glycerol production by *Eurotium amstelodami* were recorded at 25°C.

Certain metals were added as salts to the defined basal culture medium in low level (25 µmol/l) to determine their influence on mycelial growth and glycerol production by the experimental organism. Different patterns of effect were recorded with the various ions tested. The first group of elements which caused complete inhibition of growth and glycerol production by the experimental organism included copper, mercury, silver, lead and cobalt (Fig. 8). The effect of heavy metals, particularly copper and mercury on fungal cell have for long been of interest to mycologists as a result of their value as fungicides. Ross (1975) reported that chemical properties of metals can be related in a general way to their relative toxicities to fungi and higher plants. The more electronegative metals such as copper, mercury and silver have high affinities for sulphydryl, amino and imino groups which are likely to be reactive site on many enzymes.

The second group of elements which included cadmium, lithium and molybdenum, not only retarded mycelial growth but also restricted glycerol formation (Fig. 8). Bowen (1966) and Ross (1975) recorded that all the divalent transition elements as molybdenum and silver are poisonous as a result of reactivity with enzymes. It has been also found that cadmium acts on the cell membrane causing damage and changes in permeability (Passow et al., 1961).

The third group of elements comprised manganese and iron, both ions of these elements increased mycelial growth but retarded glycerol production. No effect was recorded either on mycelial growth or glycerol production by addition of nickel ions (Fig. 8).

The fourth group of elements included zinc and calcium. Addition of ions of these elements, individually, stimulated both mycelial growth and glycerol production by the experimental organism (Fig. 8). Of all the trace elements zinc seems to play a key role in the biosynthesis of many metabolites including glycerol. At least, twenty enzymes have been found to be zinc dependent (Parisi & Vallee, 1969), which may partly account for its key role. Bowen (1966) listed calcium as one of number of metals

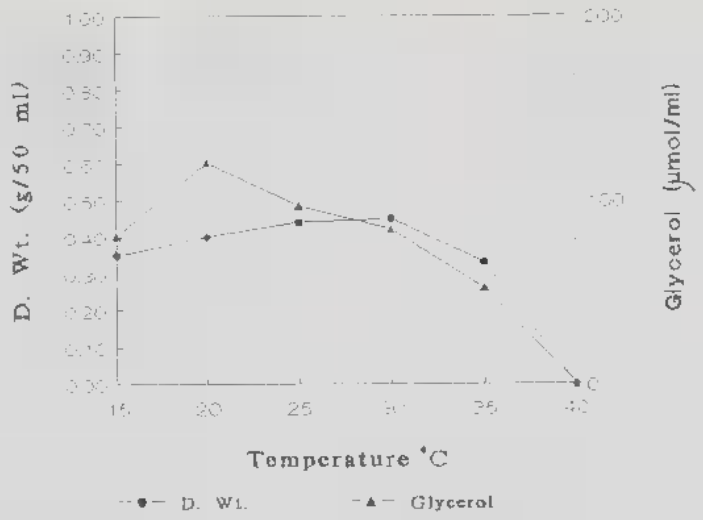

Fig. 7: Effect of temperature on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 7: Effets de la température sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

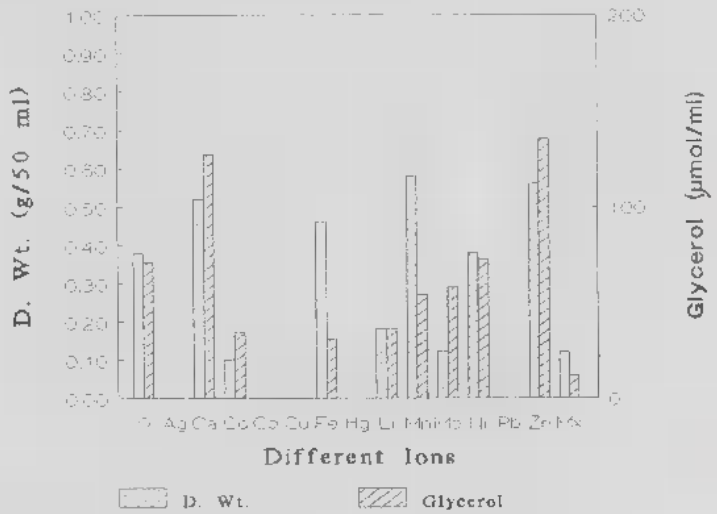

Fig. 8: Effect of different ions on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 8: Effets de différents ions sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

which proved to be essential for fungal growth. Leopolid & Willing (1984) found that $CaCl_2$ between 1 and 50 µmol served partially to protect the tissue of leaf slices from NaCl damage. Gary-Boba (1970) suggested that calcium may serve to bind phospholipids together and this limits membrane permeability. Ca^{+2} ions present in the cell wall are, to large extent, responsible for its structural and mechanical properties (Probine & Preston, 1962).

Because the C:N ratio of medium significantly affected polyalcohol production by *Pichia miso* as reported by Onishi et al. (1961), the effect of peptone, yeast extract and casamino acids concentrations on mycelial growth and glycerol formation by the experimental organism were examined. The results obtained (Fig. 9) show that increasing of the three nitrogen rich compounds concentrations gradually increase both mycelial growth and glycerol production. High concentrations (6-10 g/l) of these compounds promote growth but adversely affected accumulation of glycerol. The highest levels of glycerol were recorded in the presence of 6 g of each compounds per liter of medium (Fig. 9). In a previous study, Parekh & Pandey (1985) carried out a trial for optimization of yeast extract and corn steep liquor (CSL) as nitrogen source of culture media for glycerol production by *Hansenula anomala*. They found that yeast extract at 0.2% and CSL at 0.3% level were optima and provided roughly equivalent amounts of nitrogen. Recently Hendriksen et al. (1988) reported that the highest combined yield of mannitol and glycerol (65 g/l) was obtained with *Penicillium aethiopicum* IBT MILA4, when grown on 150 g of sucrose and 20 g of yeast extract per liter. However, Luard (1985) concluded that complex organic forms of nitrogen were not necessarily the most efficacious.

The effect of different rates of aeration on mycelial growth and glycerol formation was also studied. The results obtained (Fig. 10) show that the highest aeration condition, with 25 ml of the medium in 500 ml Erlenmeyer flasks, gave the best yield of glycerol. Increasing the amount of medium in 500 ml flasks gradually decreased glycerol production and increased mycelial growth of the experimental organism. These results are in complete harmony with that obtained by several investigators. Quantitative studies (Spender et al., 1957) of the relationship between glycerol production and rate of aeration showed that glycerol yield (by osmophilic yeast) increased with the increase of aeration rate. Dukema et al. (1985) reported that glycerol is one of the polyols that is formed rapidly by *Aspergillus nidulans* (= *Emericella nidulans*) when this fungus is grown on glycolytic carbon sources, especially under strong oxygenation. Effect of aeration on glycerol production by *Hansenula anomala* was studied by Parekh & Pandey (1985) and showed that more than 20-31 ml medium in 500 ml flasks decreased the productivity, suggesting the need for vigorous aeration. Also Onishi & Susuki (1968) recorded that the highest aeration with 30 ml and 100 ml medium in 500 ml flasks gave the best yield of mannitol and glycerol, respectively.

The results obtained during this work propose a suitable culture medium and some optimal conditions for production of glycerol which are practically suitable for routine screening of glycerol-producing fungi.

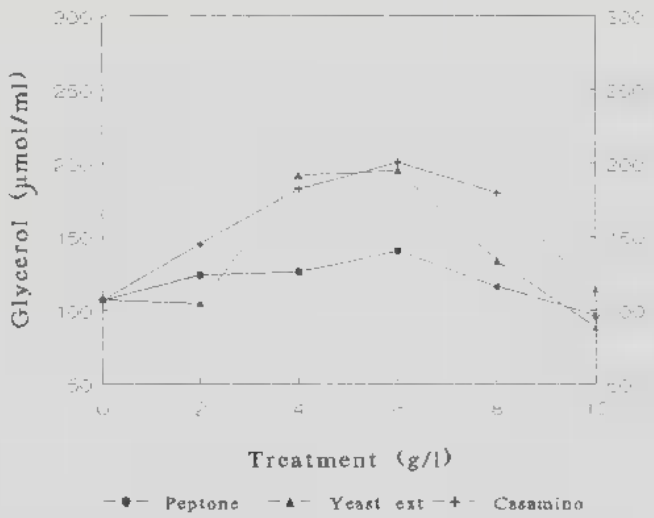

Fig. 9: Effect of different concentrations of peptone, yeast extract and casamino acids on glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 9: Effets de différentes concentrations en peptone, extrait de levure et caséine sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

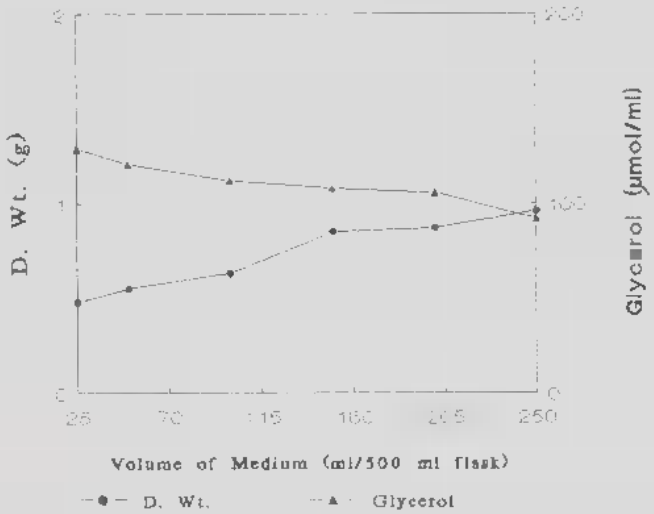

Fig. 10: Effect of different rates of aeration on the growth and glycerol production by *Aspergillus wentii* (IMI 023010).
Fig. 10: Effets de l'aération sur la croissance et la production de glycérol par *Aspergillus wentii* (IMI 023010).

REFERENCES

ABDEL-HAFEZ A.I.I., 1991 - Soil fungi of mangrove ■ Egyptian red sea coast and glycerol accumulation by four *Eurotium* species. *Bull. Fac. Sci. Assiut Univ.* 20: 137-160.

BOWEN H.J.M., 1966 - Trace elements in biochemistry. Academic Press, New York.

BROWN A.D., MACK ENZIE K.F. and SINGH K.K., 1986 - Selected aspects of microbial osmoregulation. *FEMS Microbiol. Rev.* 39: 31-36.

DUKEMA C., KESTER H.C.M. and VISSER J., 1985 - ^{13}C NMR studies of carbon metabolism in the hyphal fungus *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. U.S.A.* 82: 14-18.

EGGSTEIN M. and KUHLMANN E., 1974 - Triglycerides and glycerol. Determination after alkaline hydrolysis. *In:* Methods of Enzymatic Analysis. pp. 1825-1831. Edited by H.U. Bergmeyer. Academic Press, London.

GARY-BOBA C.M., 1970 - Effect of Ca^{+2} on the water and non-electrolyte permeability of phospholipid membranes. *Nature* 228: 1101-1102.

GOLD H.S., 1959 - Distribution of some lignicolous ascomycetes and fungi imperfecti in an Estuary. *J. Elisha Mitchell Sci. Soc.* 75: 25-28.

HENDRIKSEN H.V., MATHIASEN T.E., ADLER-NISSEN J., FRISVAD J.C. and EMBORG C., 1988 - Production of mannitol by *Penicillium strains*. *J. Chem. Tech. Biotechnol.* 43: 223-228.

HOLLIGAN P.M. and JENNINGS D.H., 1972 - Carbohydrate metabolism in the fungus *Dendryphiella salina*. II- The influence of different carbon and nitrogen sources on the accumulation of mannitol and arabitol. *New Phytol.* 71: 583-594.

INLOW D., McREAE J. and BEN-BASSAT A., 1988 - Fermentation of corn starch to ethanol with genetically engineered yeast. *Biotechnol. Bioeng.* 32 (2): 227-234.

LEOPOLID A.C. and WILLING R.P., 1984 - Evidence for toxicity effects of salt on membranes. *In:* STAPLES R.C. and TOENNISSEN G.H. (Eds.), Salinity tolerance in plants strategies for crop improvement. 227 p. Awiley-Iersicence Publication. Toronto Singapore.

LUARD E.J., 1985 - Interaction of substrate C:N ratio and osmotic potential on growth and osmoregulation of four filamentous fungi. *New Phytol.* 101: 117-132.

NEUMULLER O.C., 1981 - Glycerin. *In:* NEUMULLER O.A. (ed.) Rompps Chemie-Lexikon, Vol. 2, 8th ed. Frankch, Stuttgart, pp. 1512-1513.

ONISHI H., 1959 - Studies on osmophilic yeast: VI- glycerol production by the salt-tolerant yeasts in the medium with high concentrations of sodium chloride. *Bull. Agr. Chem. Soc. Japan* 23: 359-363.

ONISHI H. and SUZUKI T., 1968 - Production of D-mannitol and glycerol by yeasts. *Appl. Microbiol.* 16: 1847-1852.

ONISHI H., SAITO N. and KOSHIYMA I., 1961 - Studies on osmophilic yeast. XI. Various factors affecting polyalcohol production by *Pichia miso*. *Agr. Biol. Chem. (Tokyo)* 25: 124-130.

PAREK S.R. and PANDEY N.K., 1985 - Production of glycerol by *Hansenula anomala*. *Biotechnol. Bioeng.* 27: 1089-1091.

PARISI A. and VALLEE B.L., 1969 - Zinc metalloenzymes: Characteristics and significance in biology and medicine. *Am. J. Clin. Nutr.* 22: 1222-1239.

PASSOW H., ROTHSTEIN A. and CLARKSON T.W., 1961 - The general pharmacology of heavy metals. *Pharmacol. Rev.* 13: 185-224.

PETERSON W.H., HENDERSHOT W.F. and HAJNY G.J., 1958 - Factors affecting production of glycerol and D-arabitol by representative yeasts of the genus *Zygosaccharomyces*. *Appl. Microbiol.* 6: 349-357.

PITT J.I., 1975 - Xerophilic fungi and the spoilage of foods of plant origin. *In:* Food science and technology (DUCKWORTH R.B., ed.), pp. 273-307. Academic Press, London, New York.

PROBINE M.C. and PRESTON R.D., 1962 - Cell growth, the structure and mechanical properties of the wall in internodal cells of *Nitella opaca*. II- Mechanical properties of the wall. *J. exp. Bot.* 13: 111-127.

REHM H.J., 1988 - Microbial production of glycerol and other polyols. *In:* REHM H.J. and REED G. (eds.), Biotechnology, Vol. 6b. VCH Verglagsgesellschaft, Weinheim, pp. 52-69.

ROSS I.S., 1975 - Some effects of heavy metals on fungal cells. *Trans. Br. Mycol. Soc.* 64: 175-193.

ROSS S.S. and MORRIS E.O., 1962 - Effect of sodium chloride on the growth of certain yeasts of marine origin. *J. Sci. Food Agric.* 13: 467-475.

SCOTT W.J., 1957 - Water relations of food spoilage microorganisms. *Advances in Food Res.* 7: 83-127.

SPENCER J.F.T. and SHU P., 1957 - Polyhydric alcohol production by osmophilic yeasts: effect of oxygen tension and inorganic phosphate concentration. *Canad. J. Microbiol.* 3: 559-567.

SPENCER J.F.T., ROXBURGH J.M. and SALLAN H.R., 1957 - Factors influencing the production of polyhydric alcohold by osmophilic yeasts. *J. Agri. Food Chem.* 5 (1): 64-67.

VIJAIKISHORE P. and KARANTH N.K., 1986 - Process Biochem., 21: 54-57. *In:* YAMADA K., KUMAE S. and TANI Y. (1988): Derivation of a glycerol-negative mutant of ■ methylotrophic yeast, *Candida boidinii* and accumulation of glycerol in methanol medium. *Agric. Biol. Chem.* 51: 1945-1949.